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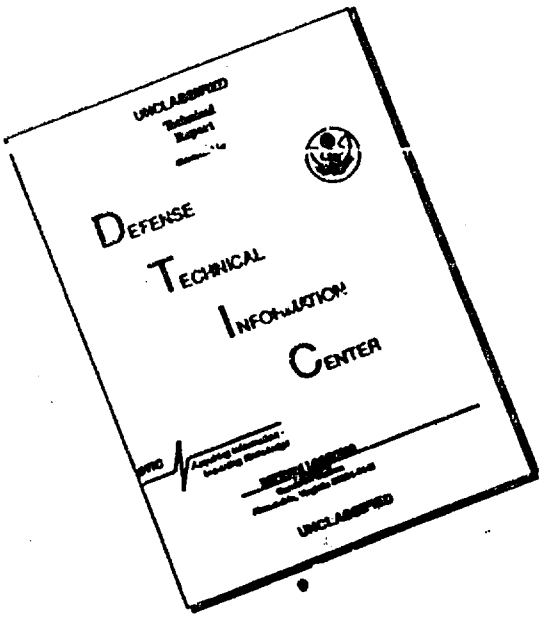

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Observations on the Mechanism of the Sensitizing Action of Botulinal Toxin

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The old dispute about botulism as to what it represents in a sense of pathogenesis may be regarded as ended today. Not only the toxinfectious nature of botulism had been indisputably proven mainly by the efforts of Soviet investigators, but also the basic factors which condition the infectious effects of Cl. botulinum in the animal organism.

Among these factors of the main importance is the botulinal toxin, which, together with the microbe, causes botulism diseases.

MINERVIN et al. indisputably proved under experimental conditions the effects of the toxin and this has been further verified by MATVEEV. A detection of this fact permitted us to substantiate a series of observations made in the field of epidemiology and to

impart clinical data on botulism, together with the laboratory findings developed in the course of investigations; it also permitted to establish new facts, of which the basic ones are the following:

1) The production of the toxin takes place in the very organism in the course of toxinfection caused by Cl. botulinum;

2) The production of the toxin originates mainly in the digestive tract;

3) The antitoxic antibotulinal serum must be administered in the course of treatment not only by parenteral, but also by the enteral method.

The question of the mechanism of the "sensitizing" activity of the toxin in infected organism assumes a great significance in view of the specific importance of the role of the toxin (as established by us) in the formation of infectious effect of the microbe, i.e. the causative agent of botulism. Due to this condition, the microorganism has an opportunity to multiply, to produce the toxin and thus to render its pathogenic effect. The explanation of this fact would be, on the one hand, a new proof that fully acknowledges the toxinfectious nature of botulism, and, on the other hand, it would permit a researcher to come closer to the clarification of the essence and characteristics of the process that unfolds itself in an organism stricken by botulism.

In selecting a trend with which we should carry on the investigation of the discussed problem, we recognized the following considerations. First, the phagocytic phylaxis is one of the basic

factors which determines the natural immunity in the organism. Many authors, beginning with van ERMENGEM, explain by phagocytosis the resistance of the organism to the spores of Cl. botulinum. But, none of the investigators studied the effects of the botulinal toxin on leukocytes and on phagocytosis.

Thus, in order to study this problem, we set our experiments in two directions: on the one hand, we studied the effects of the toxin on leukocytes and on phagocytosis in vitro, and, on the other hand, we administered the toxin to guinea pigs and then, at various times, we investigated the phagocytic activity of leukocytes in the blood of these animals.

Briefly, the experimental method boiled down to this. We used botulinal toxin type A, obtained in a usual way by cultivating botulinal strain No.98. The titrations of various series of fresh toxins ranged from 1:',000 to 1:60,000 for white mice. In some experiments we used old toxin with a titer of 1:300. We also used the golden staphylococcus strain No.209 as a culture on which we tested the phagocytic activity of leukocytes. A suspension of this microbe was prepared from a daily agar culture and it contained 2 billion microbic bodies per 1 ml.

In control experiments, instead of the active toxin, we used inactivated toxin that was heated in a water bath for 20 minutes. In two other series of the control test tubes, we substituted the toxin either by the initial broth in which the toxin was prepared, or by a physiological solution. The test tubes were kept in an incu-

bator for 30 minutes, whereupon a count of phagocytized cocci was made and included 50 leukocytes. Then, we concluded the phagocytic index and computed the average number of cocci per one leukocyte.

We resolved 7 series of investigations in a total number of 65 experiments. The area and the results of these investigations were concentrated on the following course. In the first series of investigations we sampled a freshly prepared toxin with the titer 1:60,000 and we took a human blood as a source of leukocytes.

The examination included the effects of the whole toxin, as well as the toxin in dilutions of 1:100, 1:1,000 and 1:10,000. We also examined the effects of the toxin inactivated in 30 minutes at 100°C.

Phagocytosis Test with Leukocytes of Human Blood

Ingredients added to the citrated human blood	Dilutions of toxin				Control of phagocytosis
	Whole	1:100	1:1,000	1:10,000	
Botulinal toxin	0.94	2.84	3.4	4.18	--
Inactivated toxin	4.24	4.34	4.5	3.98	--
Initial broth	5	--	4.08	5.36	--
Control of phagocytosis	--	--	--	--	4.01

The Table shows that botulinal toxin exerted a considerable effect on the intensity of staphylococci. The latter still showed a slightly inhibiting effect in a 1:1,000 solution. The specificity of the toxin's effect was apparent from this that, while depressing the effects of phagocytosis, the intensity of the toxin decreased with the extent of the toxin's dilution. This became particularly

clear from the fact that the toxin inactivated by boiling ceased to affect the phagocytosis.

The findings resulting from the second and third experiments conducted according to the same procedure and with the same blood used, differed little from the results just described. Also here the undiluted toxin revealed its maximum depressing effect on phagocytosis. And here, too, the same rules prevailed in connection with a decrease of the toxic effect on phagocytosis, i.e. in accordance with the extent of the toxin's dilution. Control experiments with the inactivated toxin and with the initial broth, as well as with the physiological solution, brought approximately the same phagocytic indices.

The next two series differed from the aforedescribed in this respect that we used a 2-month old toxin in one experiment (No.4) with its lethal dose for white mice equal to 1:300; we used in the other experiment (No.5) a fresh toxin with a titer 1:1,000 and, instead of human blood, we used rabbit's blood.

The results of the two investigations differed considerably from the results of the first three series. The difference extends to this. First of all, the degree of the suppression of phagocytosis was expressed much more sharply. Thus, in the experiment No.4, involving a whole (undiluted) toxin, the phagocytic index was only 0.06; with a fresh toxin - 0.04; the retardation still observed with a 1:20,000 dilution was 2.98, while the control index was 3.54. The doses of the toxin were smaller in both experiments than a single minimal lethal dose of a mouse, but they retarded the phago-

cytic activity of leukocytes considerably more.

The indicated difference in the results of the two groups of experiments can only be explained by this that in the first experiments we made observations on human leukocytes, while in the subsequent two groups - on rabbits' leukocytes. It is a known fact that the sensitivity to leukocidin differs with various types of leukocytes. LENGELSHEIM noticed this already in 1900. He proved that leukocytes of frogs are entirely insensitive to staphylococcic leukocidin, while mice and guinea pigs are very little sensitive, also young dogs are moderately sensitive, but rabbits are highly sensitive.

The purpose of the subsequent series of experiments in vitro was to study the problem: what will be the effect of the toxin on the phagocytic properties of leukocytes, after the toxin had been influenced by the neutralization with the antibotulinal antitoxic serum. The method of the experiments contained the following objective. We sampled two series of toxins. The minimal fatal mouse dose of one series was equal to 1:1,000, and the other - to 1:300. In these experiments we mixed 0.5 ml of the toxin with 0.2 ml of the antitoxic serum type A; then, to two other test tubes, which contained the same quantity of toxin, we added 0.2 ml of standard horse serum. The test tubes with the mixtures were placed in incubator for 1 hour, whereupon, according to the usual method, one volume of their contents was added to three volumes of the citrated rabbit's blood and, then, this was mixed with one volume of the staphylococcal suspension.

Instead of one volume of the toxin's mixture with the serum,

we used for the control test tube one volume of pure toxin, either whole, or diluted 1:100.

Following the usual 30-minute stay of the mixture in the incubator, we took smears in order to determine the phagocytic index.

Thus obtained results showed that the toxin under these conditions also had a sharp effect on phagocytosis. While the phagocytic index in the control experiment (where no toxin was used) equaled 3.02, it decreased to 0.14 and 0.18 in experiments with whole toxins, and to 0.8 and 0.86 with the toxins diluted 1:100. However, following a prior storage with the antitoxic serum, the depressive effect of the toxin decreased; the phagocytic index increased in experiments with the whole toxin to 1.14 and 1.28, also to 2.22 and 2.08 with the toxin diluted to 1:100.

In the experiment with a standard horse serum the phagocytic indices were 0.16 and 0.08 for whole toxins, and 0.68 and 0.7 for diluted toxins.

We suppose that the results of these experiments contain additional and very conclusive data in favor of this that botulinal toxin possesses in a high degree a peculiarity in that it decreases the phagocytic properties of leukocytes, and also that this characteristic of the toxin bears its highly distinct pattern to the extent that a specific antibotulinal serum can neutralize it.

The results obtained by us indicated a diverse effect of the botulinal toxin on the phagocytic activity of leukocytes and induced us to modify somewhat the experimental technique toward lengthening the contact time of the toxin with leukocytes. This problem was of

interest in connection with this that under usual experimental arrangement the effect of the toxin on leukocytes is merely extended to 30 minutes and that, in a naturally progressing toxinfection of botulism, the toxemic process can be fairly long. The change of the technique in the arrangement of our investigations was reduced to this: prior to the experiment with phagocytosis, we kept the citrated rabbit blood at 37°C for one hour either with a whole toxin, or with a toxin diluted to 1:100, and only after this we added the suspension of staphylococci. The subsequent course of the experiment followed in a usual order. For control purposes we run an experiment in which the citrated rabbit's blood without a toxin was stored for one hour at 37°C, whereupon it was used as one of the ingredients in the experiment with the phagocytosis.

While sampling one of the toxins in a whole condition, we found the phagocytic index at 0.02, then, with the same toxin diluted to 1:100, the phagocytic index was 0; the phagocytic indices with the next toxin were respectively 0 and 0.02. At the same time, when the citrated blood was stored without a toxin in an incubator, the phagocytic index in control experiment was 2.96.

The described observations showed undoubtedly that, a sharp decrease of phagocytosis, which is combined with the time of action of the toxin on leukocytes, depends precisely on the effect of the toxin and not on any other factors. Thus, we were confronted with the question: is a change in the basic biological properties of leukocytes, resulting from the botulinal toxin, accompanied by a change in the morphology of leukocytes? Yet, the investigations

proved that considerable changes in the structure of leukocytes were observed not only in the experimental but also in control smears, where the leukocytes were not exposed to the action of the toxin.

The results obtained by us during investigation of the effects of the botulinal toxin on phagocytic characteristics of leukocytes in experiments in vitro induced us to make analogous observations with relation to leukocytes of guinea pigs, after they received the type A toxin parenterally. Our experiments were disadvantageous in some respects. Since we were unable to select all animals of alike weight for our observations, the weight of guinea pigs in individual experiments varied between 200 and 800 gm. However, the results obtained according to the rules were admissible so that the circumstance mentioned by us looses even its relative importance substantially.

The main point of the observations on guinea pigs centered on this. We had 25 animals for the experiment. Prior to administration of the toxin, we took one or two blood tests of guinea pigs (from the aural vein) and, according to the aforedescribed method, we performed the phagocytosis experiment with staphylococci (strain No.209). Then, we administered the toxin in any quantities to guinea pigs, and we took the blood test again after the following fixed time periods: 30 minutes, 3 hours and every consecutive day, until death, or recovery.

One guinea pig received in advance a fatal dose of the toxin.

The determination of the phagocytic index in this guinea pig produced the following results: the index was 11.9 and 13.8 prior to infection; then, it decreased to 6.1 after 3 hours; to 1.3 after one day; to 2 after two days; it advanced to 2.4 after 3 days; then to 3.7 after 5 days; after 35 days the phagocytic index was normal and thus it equaled 10.7.

The first series of experiments were performed on 6 guinea pigs. They were administered botulinal toxin of 5-day growth (minimal lethal dose for a mouse 1:500) in nonlethal quantities (one guinea pig received 3 Dlm, two - 5 Dlm each, two - 10 Dlm each and one - 15 Dlm); 13 days later, while the guinea pigs still appeared in normal conditions, they were deliberately reinfected with fatal doses of the same toxin (30, 40 and 60 lethal mouse doses). Only one guinea pig of this group was not reinfected, because of death due to accidental reason on the 7th day after exposure to the toxin.

In 30 minutes after the first administration of the toxin, we observed in all guinea pigs some drop and, at times, a sufficiently profound drop of the phagocytic index. The phagocytic index in guinea pig No.3 dropped from 4.26 to 0.86 after the animal received subcutaneously 15 mouse doses of the toxin. The drop of the phagocytic index was still greater after 3 hours; it reached its maximum after a day to 0.04. The phagocytic index began to increase after 2 days; but even after 7 and up to 13 days it did not attain its initial magnitude.

The depth of the drop of the phagocytic index depended on a dose of the administered toxin. The decrease was the greatest in

one guinea pig which received 15 mouse doses of the toxin, and the smallest one in another guinea pig, which received 3 doses of the toxin.

The second administration of the toxin to guinea pigs took place after 13 days following the first infection, when the phagocytic index in animals was close to the initial index. While the weight of guinea pigs dropped slightly in the first week following the intoxication, it almost returned to the initial level at the time of the second administration of the toxin. We used 5 guinea pigs in the experiment. They received from 30 to 60 fatal mouse doses. One guinea pig died after 2 days, and 4 after 3 days. This time, the degree of the drop of the phagocytic index in the first three hours was either the same, or it was much sharper than after the first administration of the toxin. However, the degree of the drop was considerably greater in guinea pigs after a day following the second administration of the toxin, namely: the phagocytic index in two guinea pigs was zero, and in three other animals it varied between 0.2 and 0.34. After 2 days, the index in the latter three guinea pigs also dropped to zero (0.0 and 0.04).

Considering that botulinal toxin is a "sensitizer" not only to the microbe, but also to the toxicity (MINERVIN and KOTLYAREVSKAYA, 1936), we administered botulinal toxin to guinea pigs just once in this series. We used 18 guinea pigs in this experiment. All received fatal doses of the toxin and died after 1, 2, 3, 4 and 7 days. We observed in all animals a sharp drop in the phagocytic properties of leukocytes. The intensity of the phagocytosis depended

on a degree of the intoxication. Thus, in instances when death of animals followed after 1 to 2 days, the phagocytic index dropped to 0.0, 0.06 and 0.08 prior to death. In experiments, in which guinea pigs received smaller quantities of the toxin and their death followed after 3 to 4 and 7 days, their phagocytic index dropped respectively to 0.18, 0.24 and 0.78. It would be wrong to construe a purely mechanical interpretation that we observed in vitro the effects of botulinal toxin on leukocytes and on the processes that transpire in a living organism during botulism. At this point it is important to consider also the supplementary physiological mechanisms and their role with which we link the protection of an organism from infection. In connection with this, we would like to emphasize the critical, early,destructive effects in connective tissues and the "evident and constant reaction on the part of the reticuloendothelial system", which is particularly stressed by KUKAEV on the basis of the pathologohistological findings obtained from examinations of human cadavers, the victims of botulism.

According to our opinion, the changes made by botulinal toxin in the phagocytic properties of leukocytes in the blood, together with possible injuries to connective tissues and to the reticulo-endothelial system, develop the anatomical substrate, which explains the mechanism of the "sensitizing" effect of botulinal toxin.

At the same time, it is also necessary to mention that botulism affects the central nervous system, as clinical observations prove. In connection with this, even such physiological mechanisms cannot remain unaffected, which are linked with the inherent protection

of the organism from infection. One must also consider the disturbances in the functions of the vegetative neural system, which can be reflected in the phagocytic activity of leukocytes.

At one time we stated in empirical way that the greatest "sensitization" of the organism to the causative agent of botulism takes place after one day following the administration of the toxin. In studying the inhibition of the phagocytic properties of leukocytes under the influence of botulinal toxin administered to the organism, we discovered that the inhibition of the phagocytosis to a maximal degree is also observed in leukocytes after one day. The coinciding time periods which involve the maximal sensitivity of animals to the microbe after its sensitization by the toxin, together with the maximal simultaneous drop in the animals' phagocytic capacity, convinces us more firmly as to the correctness of the explanation that the botulinal toxin's "sensitization" mechanism has its effect on the microbic causative agent.

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